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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Antiestrogens are the most effective and widely administered therapy for the management of breast cancer. Their efficacy has been attributed to their ability to antagonize the estrogen receptor, and the presence of ER in breast tumor biopsy specimens correlates well with responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond to antiestrogens, while one in six patients with ER-negative breast tumors undergo objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical observations suggest that alternative mechanisms of estrogen action may regulate the growth and survival of breast tumors. We have provided evidence that estrogen acts independently of the known estrogen receptors, ERα and ERβ, via the G-protein coupled receptor, GPR30, to regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2001; Filardo et al, 2001). Moreover, we have shown that the antiestrogens, tamoxifen and faslodex (ICI 182, 780), also trigger GPR30-dependent regulation of this HB-EGF autocrine loop.

Dysregulation of the EGFR-to-MAP K signaling axis is a common occurrence in breast cancer (Slamon et al, 1989, Sivaraman et al, 1997). The subject of this DOD award is to investigate the relationship between GPR30 expression and MAP K activity in breast tumor biopsy specimens obtained at first diagnosis or following antiestrogen or other adjuvant therapies. The results of these studies may lead to a further refinement in assessing responsiveness to antiestrogen therapy.

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Introduction.

Tamoxifen is the most effective and widely administered drug for the treatment of breast cancer, providing improved disease-free and overall survival for approximately 40% of breast cancer patients (Gradishar and Jordan, 1998). More recently, it has been shown that tamoxifen also serves as a chemopreventive agent for certain women at high risk for developing breast cancer (Fisher et al, 1998). Its value as a therapeutic agent is presumed to be associated with its ability to function as an ER antagonist (Katzenellenbogen et al, 1997), and accordingly, the presence of ER in breast tumor biopsy specimens provides some degree of certainty in terms of predicting responsiveness to tamoxifen therapy (Witliff, 1984). However, one in four patients with ER-positive tumors (15% of all breast tumors) for whom tamoxifen is indicated, fail tamoxifen therapy; and one in six patients with ER-negative tumors (5 % of all breast tumors), for whom tamoxifen is not currently indicated, exhibit objective tumor regression (Witliff, 1984). Moreover, it is not uncommon for breast carcinomas that initially respond to tamoxifen to acquire tamoxifen resistance, and eventually fail treatment (Katzenellenbogen et al, 1997). Thus, there is an urgent need to identify patients capable of responding to tamoxifen therapy from those who require alternative treatment modalities. This is particularly true for patients with ER-negative tumors since the majority of these patients do not respond to tamoxifen, and consequently, they have fewer treatment options.

GPR30, an orphan G-protein coupled receptor, transmits intracellular signals that regulate the mitogen-activated protein kinases, Erk-1 and Erk-2 in breast cancer cells following tamoxifen treatment (Filardo et al, 2000, Filardo et al 2002, reviewed in Filardo et al, 2002). Erk-1/-2 hold particular significance for breast cancer because these kinases are commonly hyperactivated in breast carcinoma (Sivaraman et al, 1997); and they are key signaling intermediaries for both estrogen and growth factor-dependent pathways.

Body.

Our hypothesis is that low levels of GPR30 expression, or somatic mutations within GPR30, may compromise the ability of tamoxifen to regulate Erk-1/-2 activity, and correlate with nonresponsiveness to tamoxifen therapy. In the work supported by this award, we are testing this hypothesis by comparing the expression of GPR30 and Erk-1/-2 activity in archival breast biopsy specimens obtained at first diagnosis (prior to treatment) or subsequent to adjuvant therapy.

GPR30 expression in malignant and premalignant breast cancer..

To evaluate whether GPR30 serves as an indicator for assessing antiestrogen responsiveness, we have employed rabbit antibodies raised against a peptide from the C-terminus of GPR30 to evaluate the expression of GPR30 protein in human breast biopsy specimens. For this purpose, as reported last year, we generated a single pool of C-terminal peptide antibodies enriched from rabbit antiserum by protein G chromatography (pool# D01 = 150mls). To ensure their specificity for GPR30 protein, we tested their ability to recognize GPR30 protein in detergent lysates prepared from breast cancer cell

lines that either lack or express GPR30 protein. We confirmed that these peptide antibodies detected in MCF-7 and SKBR3 breast cancer cells a single 38-kDA band that closely approximates the predicted molecular mass of the mature 351 amino acid GPR30 polypeptide (Filardo et al, 2000). In contrast, these c-ter peptide antibodies detected relatively low levels of the 38 kDA molecular weight species in MDA-MB-231 cells. Upon transfection with GPR30 cDNA, MDA-MB-231 cells expressed high levels of GPR30 protein. GPR30 was not detectable in vector-transfected MDA-MB-231 cells.

Further testimony to the specificity of the C-ter GPR30 peptide antibodies was provided by the fact that soluble C-terminal GPR30 peptide competed for their reactivity to immobilized C-terminal peptide, while soluble peptide derived from the N-terminus of GPR30 demonstrated no inhibitory effect (figure 2, appendix, 2001 progress report). As we reported last year, GPR30 C-ter peptide antibodies appeared to react specifically with formalin-fixed breast cell lines that express GPR30 but were nonreactive with MDA-MB-231 breast cells that do not express GPR30 protein. Using the C-ter GPR30 antibodies, we began to measure GPR30 expression in normal normal and malignant mammary ductal epithelia. We found that uniform staining with these GPR30 antibodies in normal mammary ductal epithelia (4/4), while no GPR30 reactivity was observable in sections derived from kidney. Using these tissues and cell lines, we developed a set of uniform staining conditions for detecting GPR30 protein. We found that the subcellular localization of the antibodies to the plasma membrane was consistent with our published data demonstrating that GPR30 is associated with the plasma membrane (Filardo et al, 2002). We also reported last year that GPR30 expression was not uniform in the number of breast tumor biopsy specimens that we had evaluated. As evidenced in Figure 1, some breast tumors expressed high levels of GPR30. Tumors represented in panels A and B expressed high levels of GPR30 (+2). Lower levels of GPR30 were found in panel C (+1) and GPR30 protein is not detected in other tumor samples (0). GPR30 expression is observed in various premaligant states (figure 2), including; apocrine metaplasia (A), benign hyperplasia (B), atypical ductal hyperplasia (C), and ductal carcinoma in situ (D). In no instance have we detected a premaliganant lesion that lacks GPR30 expression, and in general, premalignant ductal epithelia are more positive for GPR30 than most tumors.

For these reasons, we were quite convinced of the specificity of our antibodies for GPR30. However, based on the possible significance of GPR30 in breast cancer, Dr. Leland DeLellis, our new Chief of Pathology, pointed out to us his concern that our GPR30 antibodies showed some reactivity with the acellular portions of the breast tissue specimens. This is evidenced in Figure 2A. His expert advice was that we generate antibodies against GPR30 that lack this background reactivity.

Generation of GPR30 antibodies more suitable for immunohistochemical analysis.
During the past year, I was invited to serve as a consultant with Dr. Jan Rosenbaum, Prinicpal Scientist within the Cardiovascular Division at Proctor and Gamble Pharmaceuticals. Dr. Rosenbaum found that rabbit antibodies directed against peptides from the N-terminus of GPR30 showed good reactivity against archival specimens containing vascular tissue. Accordingly, we used the same peptide as immunogen to test

whether we could raise GPR30 N-ter peptide antibodies suitable for immunohistochemical analysis of archival breast tumor samples. We further purified these antibodies by affinity chromatography using columns containing immobilized N-terminal GPR30 peptide. These antibodies were tested for their capacity to detect GPR30 protein as determined by Western blotting. As observed with the C-terminal peptide antibodies, the N-terminal GPR30 peptide antibodies specifically recognized an appropriate-sized, 38 kDA species in breast cell lines that varied in their GPR30 expression (data not shown). The N-terminal peptide antibodies gave improved immunohistochemical staining of GPR30 without the background that we had previously observed with the C-terminal peptide antibodies. A representative example of the staining that we observe with the affinity-purified, N-terminal GPR30 peptide antibodies is shown in figure 3.

While this has been somewhat of a setback, a higher quality GPR30 antibody and the expert assistance of Dr. DeLellis will enable us to better facilitate the goals outlined in this grant award. Dr. DeLellis shows great personal interest in this project. We were without an acting Chief of Pathology prior to his arrival this year. Dr. MaryAnne Fenton, clinical oncologist will interact with us and Dr. DeLellis, to further facilitate our study by selecting tumor samples from the Rhode Island Hopital database appropriate for analysis. Dr. DeLellis had assured us that these selected tumors will be cut, mounted and presented to us for study with little delay. He has also offered his expertise in evaluating our specimens that have been stained with GPR30 as well as P-Erk (see below). In the next twelve months, we should be able to make rapid progress regarding GPR30 expression in breast tumors.

P-Erk expression in infiltrating ductal carcinoma of the breast at first diagnosis. We have reported last year that constitutive Erk-1/-2 activation is commonly observed in primary breast carcinomas prior to tamoxifen therapy. As outlined in the statement of work for year two, we have extended our analysis of P-Erk expression in breast cancers harvested at first diagnosis (no adjuvant therapy). To date, of the 46 breast tumors that have been examined, we find phosphorylated (active) Erk-1/-2 (P-Erk) in greater than 70% of these tumors.. Consistent with our findings from last year, expression of hyperactive Erk-1/-2 in these tumors does not appear to correlate with the expression of ERα or amplification of the EGFR family member, HER-2/neu. Now that we have established During the next 12 months, serial sections from these tumors (with or without adjuvant therapy) will be further stained for GPR30 expression as outlined below.

Key Research Accomplishments.

1. **Publications, Research Scholar Award and Consults.**

   In the past twelve months, we published one primary article and a research perspective supporting a role for GPR30 in breast cancer. We were also invited to submit a chapter for a book and to serve as a consultant with Procter and Gamble. Finally, we received a Research Scholar Award from the American Cancer Society. (see Reportable Outcomes below).

2. In late December, Dr, Ronald DeLellis, was named as the new Chief of Pathology at Rhode Island Hospital. We had been without a Chief of Pathology for some time. Dr. DeLellis pointed out to us that while our GPR30 peptide antibodies appeared to be specific for GPR30, they do have a degree of background that may be unacceptable by some pathologists. Accordingly, we tried to optimize our staining conditions and used different detection systems but to no avail.

3. We begrudgingly decided to use an alternative immunogen to raise GPR30 antibodies. Similar to the experiences of Dr. Jan Rosenbaum, with whom we consult on GPR30 action in cardiovascular function, we found that affinity-purified antibodies raised against an N-terminal peptide from GPR30 provide a more specific IHC reagent for archival breast tissue samples (compare figure 2 and 3).

4. We have extended our analysis of P-Erk expression in human biopsy specimens from patients with ER-positive and –negative breast cancer (obtained prior to first diagnosis). We continue to find that greater than 70% of these breast tumors express elevated levels of phosphorylated Erk-1/-2. Moreover, in the 46 tumors that we have examined to date, we find no correlation between phosphorylated Erk expression and either ER expression or amplification of HER2/neu (table I).

5. With better antibodies for GPR30 and a better collaborative interaction between Pathology and Clinical Oncology, we have all the necessary pieces to astisfyu the aims of this award and establish whether GPR30 plays a role in breast cancer biology and treatment.

Reportable outcomes.

1. Publications:

During the past year (second year of this award), we have published a second manuscript and one review article which support a role for GPR30 as an alternative estrogen receptor expressed in breast cancer cells. This paper further develops the hypothesis that GPR30 regulates the EGFR-to Erk signaling axis. In this most recent paper, we delineate the mechanism by which GPR30 attentuates signaling through the EGFR In the past year, we were also invited to submit a chapter for a book on membrane-associated steroid hormone receptors.

Manuscripts:

Filardo, EJ, Quinn, JA, KI Bland, and Frackelton, AR, Jr. (2000). Estrogen-induced Activation of Erk-1 and Erk-2 Requires the G-Protein-Coupled Receptor Homologue, GPR30, and Occurs via Transactivation of the EGF Receptor Through Release of HB-EGF. Molec Endocrinol. **14(10):** 1649-1660.

Filardo, EJ, Quinn, JA, Frackelton, AR, Jr. and KI Bland. Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAP K signaling axis. Molec Endocrinol. **16(1):** 70-84.

Research perspective. By invitation, I submitted a research perspective regarding the potential significance of GPR30 in breast cancer.

Filardo, E.J. (2002). Epidermal Growth Factor Receptor (EGFR) Transactivation by Estrogen via the G-Protein_Coupled receptor, GPR30: a Novel Signaling Pathway with Potential Significance for Breast Cancer. J. Steroid Biochem & Molec Biol. **80:** 231-238.

Book chapter. I was invited to write a chapter regarding our work for a book edited by Cheryl S. Watson entitled, *Membrane-associated Steroid Hormone Receptors*

Filardo EJ, Quinn JA, and Graeber CT. Evidence supporting a role for GPR30, an orphan member of the G-protein –coupled receptor superfamily, in rapid estrogen signaling.

2. Research Award

We have received a Research Scholar Award from the American Cancer Society (July 2002- June 2006) entitled "Estrogen Signaling via GPR30". With certainty, our ability to write and receive this ACS award was a direct result of the Career Development Award supporte by the DOD. The ACS grant award will enable us to further investigate the mechanism by which GPR30 transactivates the EGFR. It is complementary in nature to the studies funded by the DOD to examine GPR30 expression in human breast biopsy specimens.

3. Invited Consultant on GPR30 action.

By invitation of Dr. Jan Rosenbaum, Ph.D., Principal Scientist, Cardiovascular Research, Procter & Gamble Pharmaceuticals, I have been invited to act as a consultant regarding a possible role of GPR30 in cardiovascular disease. One direct by-product of our interaction with PGP is that we have refined our immunohistochemical staining procedures using GPR30 peptide antibodies in archival breast biopsy specimens (see Reportable Outcomes below).

Conclusions.

The known estrogen receptors, ERα and ERβ, are the best prognostic indicators for determining responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond favorably to anti-estrogens, while one in six patients with ER-negative tumors exhibit objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical findings, in conjunction with data demonstrating that antiestrogens trigger rapid signaling events typically not associated with known ERs (Aronica et al, 1994; Lee et al, 2000; Filardo et al, 2000), raises the possibility that antiestrogens may, in part, exert their antitumor effects via non ER-dependent mechanisms.

It has long been suspected that other receptors, distinct from the ER, may participate in estrogen signaling. However, until recently the physical identity of these receptors has remained unknown. Within the past two years, we have provided data demonstrating that the G-protein coupled receptor, GPR30, acts independently of known ERs to transmit intracellular signals that regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2001; Filardo et al, 2001). This signaling axis holds particular significance for breast cancer in that it is frequently hyperactivated in breast cancer. Since antiestrogens also act as GPR30 agonists that regulate EGFR-to-MAP K signaling, the studies designed here will enable us to further determine whether there is a link between GPR30 expression, Erk hyperactivation and antiestrogen responsiveness.

References.

Filardo, EJ, Quinn, JA, Bland KI, and Frackelton, AR, Jr. (2000). Estrogen-induced Activation of Erk-1 and Erk-2 Requires the G-Protein-Coupled Receptor Homologue, GPR30, and Occurs via Transactivation of the EGF Receptor Through Release of HB-EGF. Molec Endocrinol. **14:** 1649-1660.

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Wittliff JL (1984) Steroid-hormone receptors in breast cancer. Cancer **53:** 630- 643

Appendices.

A. Figures

Figure 1. Detection of GPR30 in invasive ductal and lobular carcinoma of the breast.

Representative examples of archival, formalin-fixed breast biopsy specimens obtained from patients with invasive ductal (A,C, and D) or lobular (B) carcinoma that were immunostained with rabbit peptide antibodies raised against a C-terminal peptide from GPR30. Rabbit GPR30 C-terminal peptide antibodies were used at a 1: 2, 000 dilution and were visualized using biotinylated anti-rabbit immunoglobulin, avidin-conjugated horseradishperoxidase, and diaminobenzidine as substrate. A standard set of staining conditions were employed, however notice the differences in the degree of GPR30 reactivity between the samples. A and B demonstrate the greatest degree of staining and were scored (+2). Slightly less reactivity is observed in tumor C (+1). Tumor D is negative for GPR30.

Figure 2. Detection of GPR30 in preinvasive breast cancer.

Rabbit GPR30 C-ter peptide antibodies were used to immunostain archival breast biopsy specimens obtained from patients with apocrine metaplasia (A), benign ductal hyperplasia (B), fibrocystic atypical ductal hyperplasia (C), or ductal carcinoma in situ (D). Each of these samples exhibited a higher degree of GPR30 reactivity (+3) than seen in the tumor samples shown in figure 1. Note that while there appears to be strong staining of the ductal epithelia, there is some background staining of acellular regions in these samples. This staining was not apparent with preimmune antiboides from the same rabbits. Based on this observation, it was suggested by our new Chief of Pathology at Rhode Island Hospital, Dr. Leland DeLellis, that we generate antibodies that lack this background reactivity.

Figure 3. Detection of GPR30 in normal mammary ductal epithelia employing affinity-purified antibodies raised against peptides from the N-terminus of GPR30. Following our lead, Dr. Jan Rosenbaum, with whom we consult with at Procter & Gamble Pharmaceuticals, shared with us her observation that peptide antibodies raised against the N-terminus of GPR30 exhibit more specific reactivity in formalin-fixed samples containing vascular tissue. Here, we show representative examples of normal mammary epithelia immunostained with affinity-purified rabbit antibodies from the N-terminus of GPR30 that were raised in our laboratory. The sample in (A) has been stained with preimmune rabbit antibodies, while the adjacent serial section (B) has been stained with affinity-purified N-ter peptide antibodies. (C and D) are higher magnifications of the same two samples. The tissue specimen has been counterstained with hematoxylin.

Notice that the background staining of acellular portions of the mammary tissue observed in figure 2 has been eliminated.

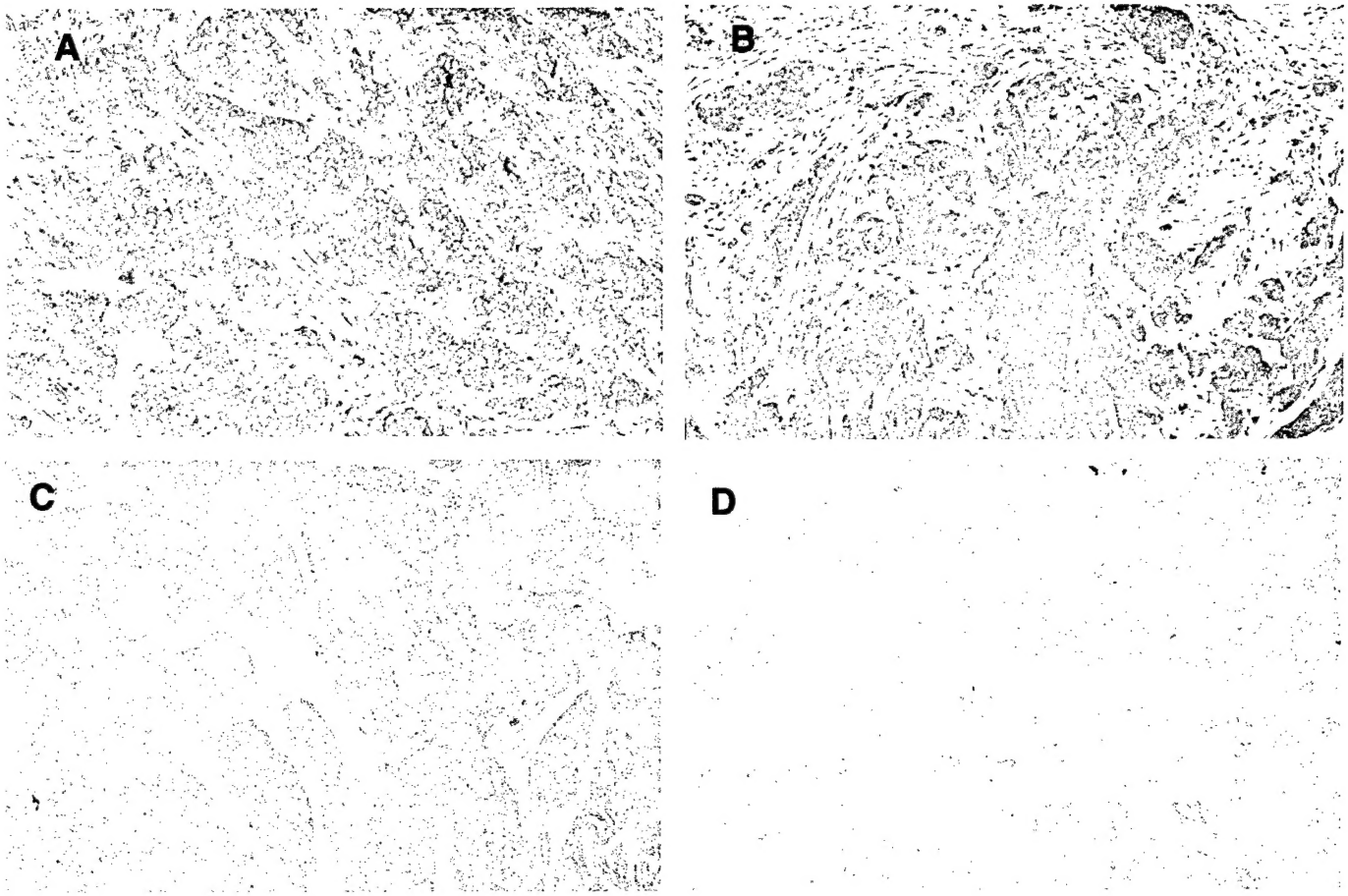
B. Manuscripts

Filardo EJ, Quinn JA, Frackelton AR Jr, Bland KI. Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol. 2002 Jan;**16(1):**70-84.

Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol. 2002 Feb;**80(2):**231-238.

C. Book chapters

Filardo EJ, Quinn JA, and Graeber CT. Evidence supporting a role for GPR30, an orphan member of the G-protein –coupled receptor superfamily, in rapid estrogen signaling in Membrane-associated Steroid Hormone Receptors. edited by Cheryl S. Watson



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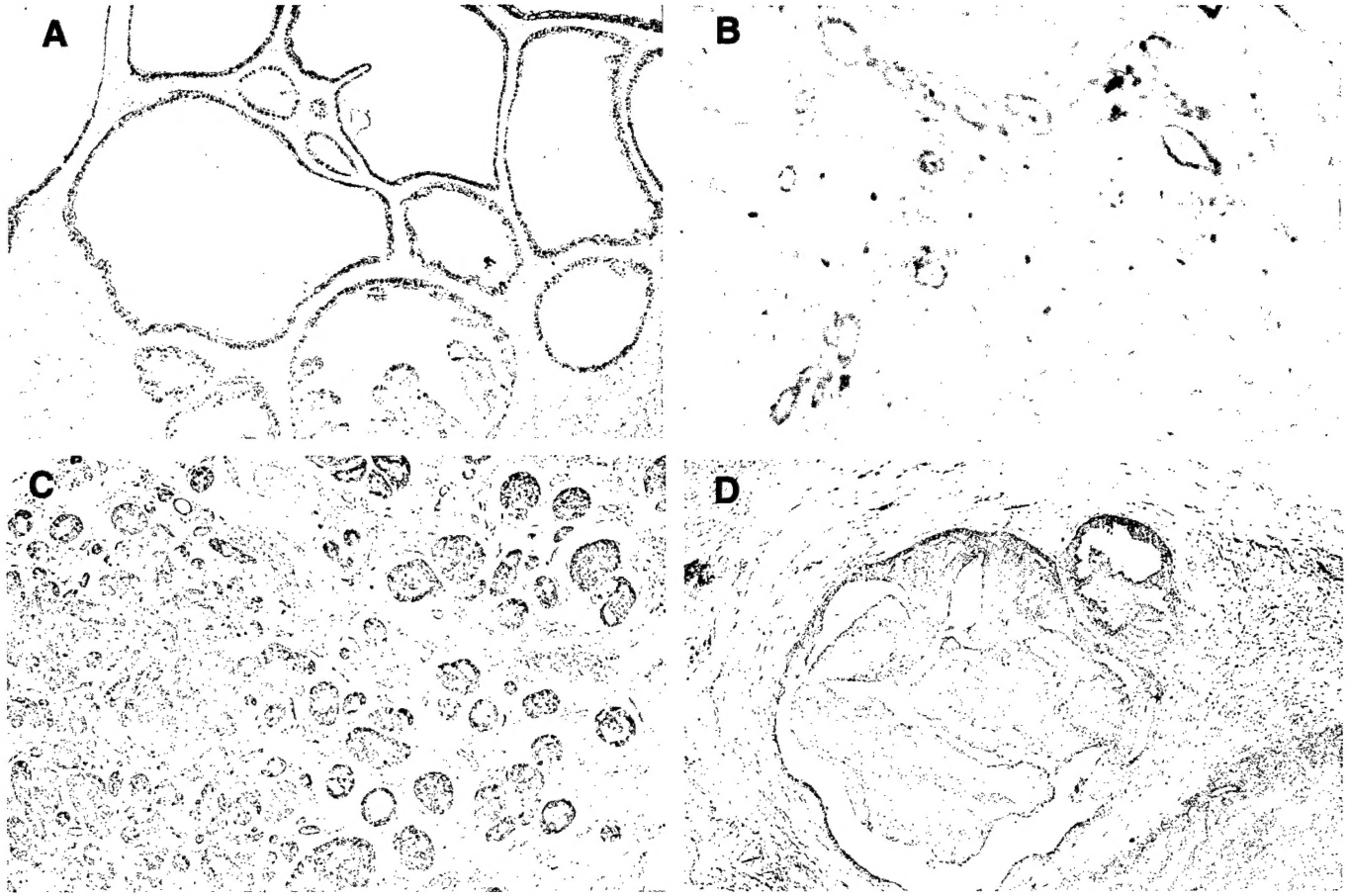

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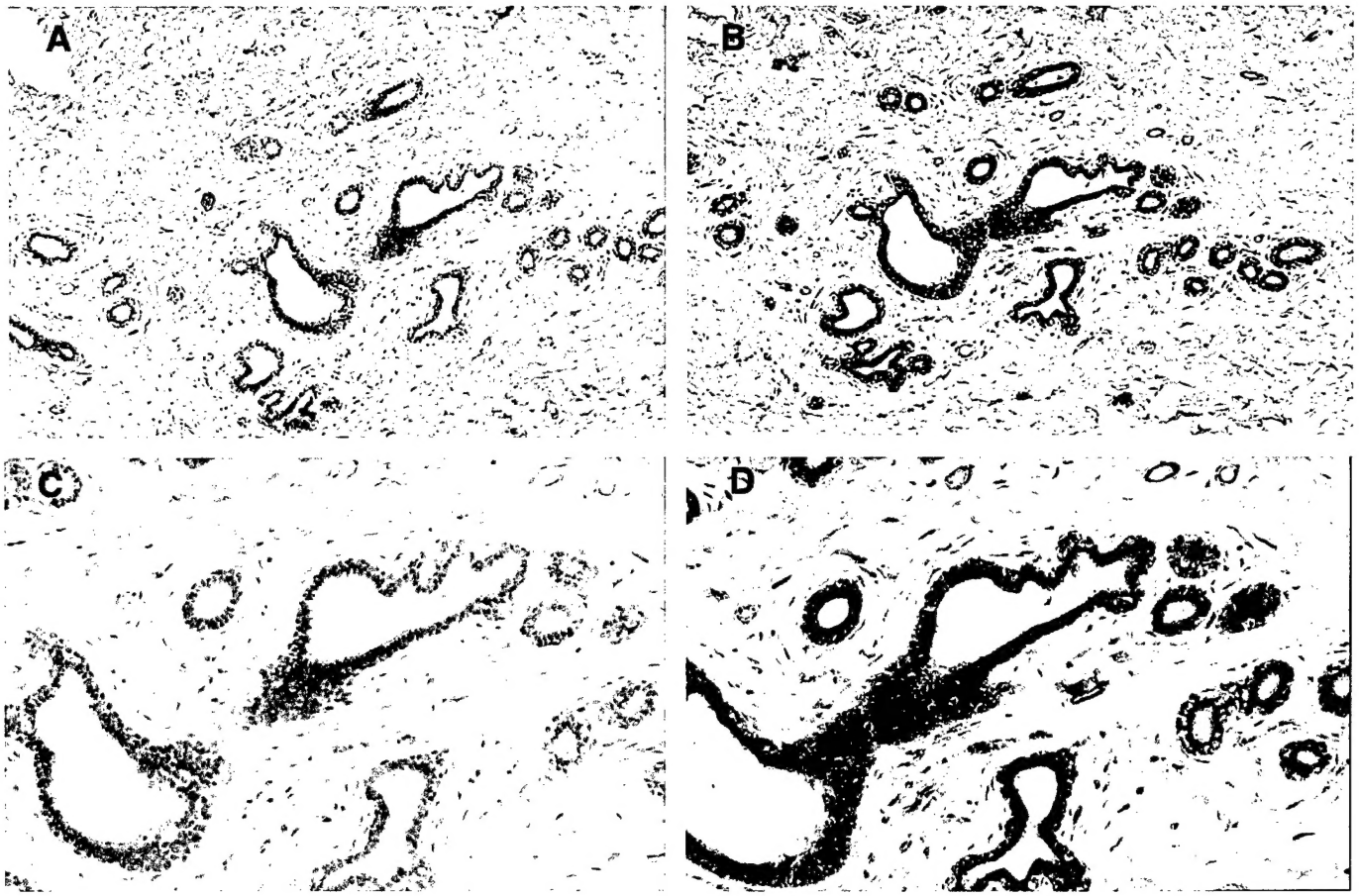

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